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Immunology

Deletion of the mouse T-cell receptor β gene enhancer blocks αβ T-cell development

[gene targeting/cis-regulatory element/V(D)J recombination/transcription]

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ABSTRACT **Intrathymic T-cell development requires temporally regulated rearrangement and expression of T-cell receptor (TCR) genes. To assess the role of the TCR β gene transcriptional enhancer (Eβ) in this process, mouse strains in which Eβ is deleted were generated using homologous recombination techniques. We report that mice homozygous for the Eβ deletion, whether a selectable marker gene is present or not, show a block in αβ T-cell development at the $CD4^-CD8^-$ double-negative cell stage, whereas the number of $\gamma\delta^+$ T cells is normal, few $CD4^+CD8^+$ double-positive thymocytes and no $\alpha\beta^+$ T cells are produced. DNA-PCR and RNA-PCR analyses of thymic cells from homozygous mutants showed no evidence of TCR β gene rearrangement although germ-line Vβ transcripts were detected at a low level; in heterozygous T cells, the targeted allele is not rearranged. Thus, deletion of Eβ totally prevents rearrangement, but not transcription, of the targeted β locus. These data formally establish the critical role played by Eβ in cis-activation of the TCR β locus for V(D)J recombination during αβ T-cell development.**

T-cell development in the thymus leads committed progenitors to two subsets of mature T cells, bearing on their cell surface clonally variable αβ or γδ T-cell receptor (TCR) heterodimers associated with the invariant CD3 polypeptides (1). Differentiation along the αβ and γδ lineages requires the assembly of the corresponding TCR variable region genes by means of a series of site-specific DNA recombinations involving variable (V), diversity (D) (in the case of the TCR β and δ genes only), and joining (J) gene segments. V(D)J recombination in immature T (and B) cells is mediated by a recombinase machinery that demands both RAG-1 and RAG-2 gene products to be active (2).

In the adult, most developing thymocytes belong to the αβ cell lineage. TCR β gene assembly (i.e., Dβ → Jβ and subsequent Vβ → DJβ rearrangements) occurs in a subpopulation of immature $CD4^-CD8^-$ [double-negative (DN)] thymocytes expressing the interleukin-2 receptor α chain (CD25) (3). The expression of a productively rearranged β gene conditions the formation of an immature form of CD3–TCR complex (termed pre-TCR) in which a TCR β-chain associates with the pre-TCR α (pTα) chain encoded by a nonrearranging gene (4). In normal mice, DN $CD25^+$ thymocytes expressing this complex are selected to develop further, leading to CD25 down-modulation, maturation into $CD4^+CD8^+$ [double-positive (DP)] cells via intermediates expressing a low level of either CD4 or CD8 and cellular expansion (5). Simultaneously, TCR β gene rearrangement is arrested to achieve allelic exclusion, whereas TCR α (Vα → Jα) rearrangement is initiated as DN $CD25^-$ cells progress toward the DP cell stage. Repertoire selection allows a small fraction of DP TCR $\alpha\beta^+$ thymocytes to survive and mature into either $CD4^+CD8^-$ or $CD4^-CD8^+$ [mature single-positive (SP)] cells, which will colonize peripheral lymphoid organs (1, 6).

We and others have previously reported that transcriptional enhancers of the TCR β and/or α genes confer lineage- and developmental-stage specificity on V(D)J recombination events within transgenic substrates, suggesting that such cis-acting regulatory elements play important roles in the control of αβ T-cell development (7, 8). However, the function of these elements at the endogenous gene level remains to be evaluated. Here we have used homologous recombination in mouse embryonic stem (ES) cells to produce mice in which the TCR β enhancer (Eβ) is deleted; the effects of the deletion on lymphoid-cell differentiation, β gene rearrangement, and transcription are described.

MATERIALS AND METHODS

Targeted Deletion of the Eβ Enhancer in ES Cells and Establishment of Eβ Deleted Mice. The targeting vector (pGΔEβ-neo; see Fig. 1*A*) was constructed by removing a 560-bp *Hpa*I–*Nco*I fragment containing Eβ (9) from a 4.95-kb *Bam*HI–*Eco*RI TCR β genomic fragment of B10.WR7 origin subcloned into pGEM-4Z (Promega), and inserting by blunt-end ligation a 1.4-kb *Xba*I–*Sal*I *Lox*P-neo-*Lox*P cassette from the pLZ neo^r plasmid (a gift from K. Rajewsky, Institute for Genetics, University of Cologne, Germany). A thymidine kinase (TK) expression cassette [a 1.8-kb *Sal*I–*Xho*I fragment from herpes simplex virus (HSV)-TK] was added onto the longest arm of homology using the *Sal*I site in the pGEM-4Z polylinker. Linearized pGΔEβ-neo vector (30 μg) was electroporated into 3×10^7 E14.1 ES cells (10) that were then cultured in the presence of G418 (200 μg/ml) and gancyclovir (2 μM). Resistant colonies carrying the intended mutation were identified by Southern blot screening using the 3′ probe (see Fig. 1*A*), further analyzed using Cβ2- and neo^r-specific probes, and then transfected with plasmid pIC-Cre (11). Cre-mediated recombination was confirmed by Southern blot analysis. To generate chimeric mice, selected ES cells were injected into BALB/c blastocysts. Male chimera were mated with either BALB/c or 129/Ola females; offspring were phenotyped by Southern blot analysis.

Flow Cytometry, Cell Sorting, and Antibodies. Flow cytometry analysis and cell sorting were performed as described (12). Fluorescein isothiocyanate-, phycoerythrin, or biotinylated-conjugated monoclonal antibodies (mAbs) against CD4 (RM4-5), CD8α (53-6.7), CD25 (7D4), CD3ε (145-2C11), αβ (H57-597), and γδ (GL3) were from PharMingen.

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Abbreviations: TCR, T-cell receptor; DN, double negative; DP, double positive; SP, single positive; ES, embryonic stem; TK, thymidine kinase.

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Immunohistology. Thymus cryosections (7 μm) were incubated with rat mAbs against cortical (CDR1; ref. 13) or medullary (95; M.N., M. Aurrand-Lions, J. DeKoning, M. Maussen, F. Galland, D. Lo, and P.N., data not published) epithelial cells, then with a peroxidase-coupled, goat anti-rat IgG + IgM $F(ab')_2$ antiserum (Jackson Immunotech, Marseille, France.); peroxidase staining was revealed using the DAB-enhancing kit (Dako). Dehydrated sections were visualized using an Axiophot microscope (Zeiss).

DNA-PCR, RNA-PCR [Reverse Transcription (RT)–PCR] Assays, and Oligonucleotides. DNA and RNA PCR assays were carried out as described (14, 15); comparative RT-PCR analyses were performed on cDNAs synthesized simultaneously. The oligonucleotides used as 5′ primers (Dβ2, Vβ5LV, Vβ5V, Vβ11LV, Vβ12, Vβ14), 3′ primers (Vβ5, Vβ11, Jβ2, Cβ2B), or hybridization probes (Vβ5V, Vβ11V, Cβ2A) have been described (7, 14, 15).

RESULTS

Production of Mice with a Deletion of the Eβ Enhancer. The mouse Eβ enhancer lies within a 560-bp *Hpa*I–*Nco*I DNA fragment located 5.5 kb downstream of the Cβ2 gene exons (9). A two-step gene targeting/Cre-mediated recombination approach (11) was used to delete this fragment in mouse ES cells (Fig. 1*A*). In a first step, the replacement of Eβ by the neomycin resistant gene (neo^r) flanked by two *Lox*P sites was achieved by homologous recombination of the targeting vector pGΔEβ-neo into E14.1 ES cells (10). Recombined clones were obtained at a frequency of ≈1/100 as indicated by Southern blot assays. In a second step, the Cre recombinase was transfected into cells from one selected clone (1B1) to remove the neo^r gene, leaving a single *Lox*P site (34 bp) at the site of recombination. Neo^r deleted clones represented ≈10% of the cells that grew up after transfection; one such clone (1B1-A3) was retained to generate chimeric mice.

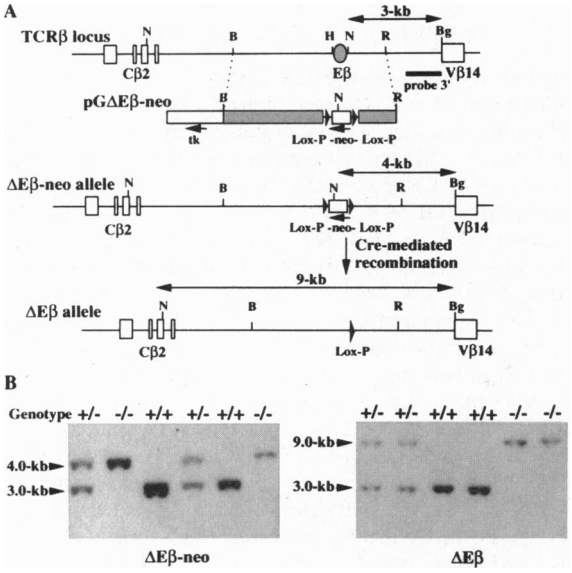

FIG. 1. Deletion of the Eβ enhancer in mouse ES cells and Southern blot analysis of the mutant mice. (*A*) Experimental strategy. Homologous recombination between the endogenous TCR β gene (top; a partial structure of the TCR β locus 3′ region is shown) and the targeting vector pGΔEβ-neo (upper middle row) produced a modified ΔEβ-neo allele. A ΔEβ allele was obtained following Cre-mediated recombination. B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; H, *Hpa*I; N, *Nco*I. The shaded boxes indicate the TCR β homologous regions used in the targeting vector; neo, a TK-neo^r cassette flanked by two *Lox*P sites (solid triangles); tk, HSV-TK cassette. The arrows indicate the transcriptional orientation of the neo^r and tk genes. The location of the 3′ probe (a 1.7-kb *Hin*dIII–*Bgl*I fragment) is indicated. (*B*) Southern blot analysis showing genotypes of mice from ΔEβ-neo (*left*) and ΔEβ (*right*) heterozygous matings. Tail DNA (10 μg) was digested with *Nco*I + *Bgl*II and assayed for hybridization with the 3′ probe. The wild-type 3-kb and mutated 4-kb (ΔEβ-neo) and 9-kb (ΔEβ) fragments as well as the deduced genotype of each animal are indicated.

ES cell clones 1B1 and 1B1-A3 were injected into host blastocysts, and the resulting chimeric mice were bred to generate heterozygous progeny carrying the Eβ replacement (ΔEβ-neo$^{+/-}$) or deletion (ΔEβ$^{+/-}$) in the germ line, respectively. Intercrosses between heterozygous animals gave birth to homozygous mutants (ΔEβ-neo$^{-/-}$ or ΔEβ$^{-/-}$) at the expected Mendelian frequency. Southern blot analysis of the different genotypic categories are shown in Fig. 1*B*. When housed under pathogen-free conditions, homozygous mice exhibited normal weight and size and were indistinguishable from wild-type and heterozygous littermates for at least 10 months. Homozygous males and females proved to be fertile.

Defective T-Cell Development in Eβ Deleted Mice. Lymphoid organs (including the thymus, spleen, and mesenteric lymph nodes) from (+/−) mice showed no evidence of gross morphological abnormalities when compared with those in wild-type littermates. However, the thymus and, to a lesser extent, the lymph nodes of both ΔEβ-neo$^{-/-}$ and ΔEβ$^{-/-}$ mice had reduced cell numbers. In 4-week-old mice, the numbers of thymocytes varied among individuals between 5×10^6 and 41×10^6, thus ranging from 60- to 7-fold less relative to those in littermate controls ($296 \times 10^6 \pm 58$ on average). No significant differences were observed between mice harboring either the ΔEβ-neo or ΔEβ mutation; this holds true for other phenotypic traits reported below and, hereafter, only results from the latter animals will be shown.

When analyzed by two-color flow cytometry using a panel of specific mAbs, lymphoid cell subsets in wild-type and ΔEβ$^{+/-}$ mice were indistinguishable; in contrast, striking differences were found when comparing cell populations from wild-type versus ΔEβ$^{-/-}$ littermates. Examples of such analysis are shown in Fig. 2. In ΔEβ$^{-/-}$ thymuses, the relative proportions of DN, CD25$^+$ (not shown), and CD3ε$^+$γδ$^+$ thymocytes were increased, whereas DP, SP, and CD3ε$^+$αβ$^+$ thymocytes were all proportionally underrepresented. In addition, staining with anti-CD4 and CD8 mAbs were lower in intensity in ΔEβ$^{-/-}$ animals as compared with wild-type controls, most probably because of the developmental arrest in these mice (see below). Finally, a similar weak staining with anti-TCR αβ (H57–597) mAb was observed on few thymocytes (and lymph node cells) from both ΔEβ$^{-/-}$ and TCR β deficient mice (i.e., mice that lack TCR β chains as a result of a 15-kb genomic replacement event that encompassed most Jβ and both Cβ1 and Cβ2 constant gene segments; refs. 16 and 17), strongly suggesting that this staining does not reflect expression of functional β chains (Fig. 2 and G.B., unpublished results); this point was demonstrated unequivocally by further PCR analysis of ΔEβ$^{-/-}$ cells purified by cell sorting (see below).

From double or triple stainings and thymocyte numbers, we calculated the absolute numbers of cells in the various thymocyte subsets (Table 1). The data indicated that the Eβ deletion did not affect DN and γδ$^+$ thymocytes, which were in normal (or slightly increased) numbers in ΔEβ$^{-/-}$ thymuses; likewise, DN CD25$^+$ thymocytes were found in normal (or slightly lower) numbers as compared with those in littermate controls. In contrast, numbers of ΔEβ$^{-/-}$ DP, SP, and CD3ε$^+$αβ$^+$ thymocytes were all dramatically reduced. Consistently, lymph node cells of ΔEβ$^{-/-}$ mice were found to contain few SP cells with a low expression of the CD4 and CD8 markers, whereas essentially all CD3ε$^+$ T cells belonged to the γδ lineage; numbers of sIgM$^+$B220$^+$ cells were slightly increased (≈1.3–2 fold) (not shown). Altogether, the above results lead us to conclude that, at the homozygous state, the Eβ deletion interferes with the differentiation of intrathymic αβ precursors

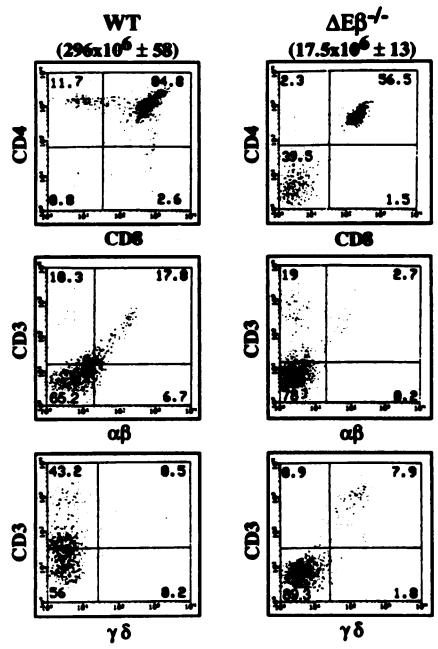

FIG. 2. Cytofluorometric analysis of thymocytes from 4-week-old wild-type or $\Delta E\beta^{-/-}$ littermates. Thymocytes were analyzed by two-color flow cytometry for the expression of CD4 versus CD8 (*top*), CD3ε versus TCR αβ (*middle*), and CD3ε versus TCR γδ (*bottom*). Forward and side scatters were used to gate out dead cells; for each sample, 2×10^3 gated events were acquired. The percentages of cells found in each quadrant are indicated; average numbers of thymocytes (from five mice of each genotype) are shown above the corresponding dot displays.

beyond the $CD25^+$ DN cell stage leading to a major defect in αβ T-cell development.

Intrathymic T-cell ontogeny involves coordinated cellular interactions between developing thymocytes and stromal cells from the thymic cortex and medulla. In parallel, differentiation of the thymic stroma requires interactions with thymocytes; cortical and medullary cell differentiation being dependent on physical interactions with early DN thymocytes and thymocytes expressing fully assembled CD3–TCR αβ complexes, respectively (1, 18). To assess the possible consequences of the Eβ deletion on these processes, we analyzed thymic sections using mAbs specific for cortical or medullary epithelial cells. As shown in Fig. 3, both types of epithelial cells were found in $\Delta E\beta^{-/-}$ thymuses. However, the size of the medulla was significantly reduced and contained few compacted cells; cortical cells were normally organized, although more densely packed than in control thymus. Therefore, developmental features of thymic stromal cells and thymocyte subsets were consistent in $\Delta E\beta^{-/-}$ mice: a relatively normal cortex correlated with unaltered early DN cell differentiation, whereas an

Table 1. Absolute numbers of thymocytes with different phenotypes ($\times 10^{-6}$)

Phenotypes	Wild type	$\Delta E\beta^{-/-}$
$CD4^-CD8^-$	5.3 ± 1.9	5.0 ± 1.9
$CD4^-CD8^-CD25^+$	2.6 ± 0.3	2.0 ± 0.4
$CD4^+CD8^+$	282.7 ± 74.7	6.0 ± 2.9
$CD4^+CD8^-$	40.8 ± 7.0	0.5 ± 0.1
$CD4^-CD8^+$	9.4 ± 2.0	0.2 ± 0.04
$CD3^+\alpha\beta^+$	65.0 ± 20	1.6 ± 1.2
$CD3^+\gamma\delta^+$	2.4 ± 1.0	2.3 ± 1.0

Numbers were obtained from three different mice of each genotype; in $\Delta E\beta^{-/-}$ mice, $CD3^+\alpha\beta^+$ corresponds to $CD3\varepsilon^+$ cells that costained weakly with the H57-597 anti-TCRαβ mAb (see text).

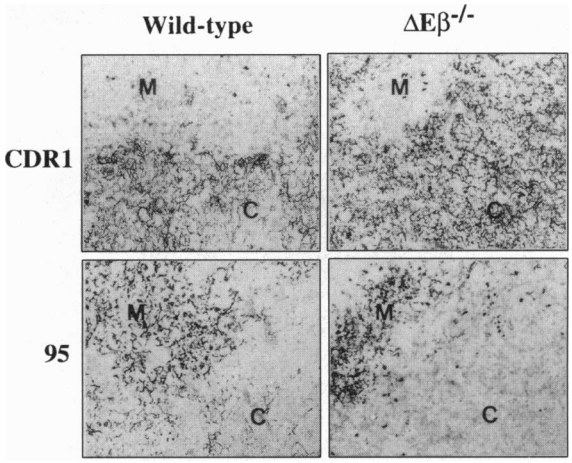

FIG. 3. Immunohistological analysis of thymuses from 4-week-old wild-type or $\Delta E\beta^{-/-}$ littermates. Binding of rat mAbs to cortical (CDR1) or medullary (95) epithelial cells was revealed by peroxidase staining. C, cortex; M, medulla. (×16.)

atrophied medulla correlated with a drastic reduction of the DP/SP cell compartments.

TCR β Gene Rearrangement and Expression in Eβ Deleted Mice. The block in αβ T-cell development observed in the $\Delta E\beta^{-/-}$ mice is most likely due to a defect in functional TCR β chains. To determine the impact of the Eβ deletion on the occurrence of β gene rearrangement, the status of the TCR β loci was analyzed in lymphoid cells of the mutant mice using DNA-PCR assays, which provide semi-quantitative information on the extent of individual Dβ → Jβ and Vβ → DJβ rearrangements (Fig. 4). When using primer combinations designed to amplify Dβ2–Jβ2 germ-line and rearranged fragments or Vβ12–DJβ2 rearranged fragments, rearrangement was not detected in thymuses from homozygous $\Delta E\beta^{-/-}$ mice (Fig. 4*B*, middle panels). According to serial dilution analyses of wild-type thymocytes (Fig. 4*B*, left panels), we estimated that there was at least 100-fold fewer rearranged products in the case of the $\Delta E\beta^{-/-}$ mice. Identical results were obtained when the Dβ1–Jβ1 cluster was analyzed in similar PCR assays, or when Vβ5 and Vβ14 specific 5′ primers were tested in combination with the Jβ2 3′ primer (not shown). It is worth noting that the levels of rearranged products were lower in heterozygous $\Delta E\beta^{+/-}$ thymocytes as compared with those in wild-type controls, consistent with an inhibitory effect of the Eβ deletion on cis-recombination. To confirm this point and evaluate the extent of inhibition in $\Delta E\beta^{+/-}$ T cells, we interbred $\Delta E\beta^{-/-}$ males (in which the targeted deletion is associated with alleles of the 129/Ola genetic background) with SJL females. SJL mice carry a > 60-kb deletion within the TCR β locus and consequently lack several Vβ genes, including Vβ12. Therefore, in the heterozygous F1 progeny, the Vβ12 gene was present on the Eβ-deleted allele only. Significantly, Vβ12–DJβ2 rearrangements, which were found in thymocytes and lymph node cells from wild-type animals, were not detected in cells from ($\Delta E\beta^{-/-}$ × SJL) offspring (Fig. 4*C*, left); as a control, the Vβ14 gene that is in both 129/Ola and SJL genetic backgrounds was found to be rearranged in the later cells (Fig. 4*C*, right).

Finally, because flow cytometric analyses detected a few TCR $\alpha\beta^{low}$, $CD8^{low}$, and/or $CD4^{low}$ SP cells in $\Delta E\beta^{-/-}$ mice (see above), we analyzed cells purified by cell sorting from $\Delta E\beta^{-/-}$ thymus and lymph nodes including: (*i*) $CD8^+$ SP cells, (*ii*) $CD4^+$ SP cells, and (*iii*) $CD3\varepsilon^+$ cells that costained weakly with the H57-597 anti-TCR αβ mAb. Neither Dβ → Jβ nor Vβ → DJβ rearrangements were detected in any of these cell populations (Fig. 4*B*, right panels; only the $CD8^+$ data are

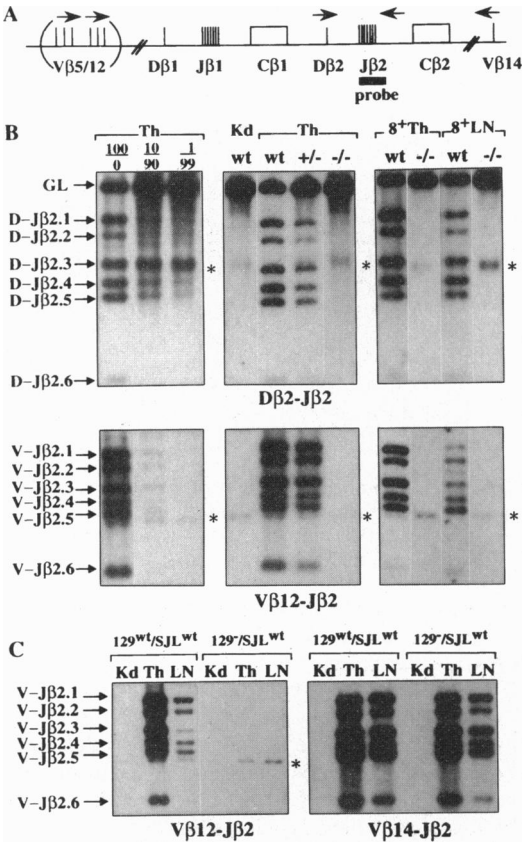

FIG. 4. Relative levels of TCR β rearrangement in lymphoid tissues and cells from wild-type (wt) and mutant $\Delta E\beta^{+/-}$ or $\Delta E\beta^{-/-}$ mice. The extent of Dβ → Jβ and Vβ → DJβ rearrangements was analyzed by DNA-PCR using genomic DNA from 2×10^3 thymocytes (Th), lymph node cells (LN), or $CD8^+$ SP cells purified by cell sorting from thymus and lymph nodes (8^+Th and 8^+LN, respectively); DNA (10 ng) from kidney (Kd) was used as a source of TCR β germ-line DNA. (*A*) Diagram depicting the relative locations of the PCR primers and of the Jβ2 specific probe (a 2.3-kb *Eco*RI fragment) within the TCR β locus; the brackets schematize the genomic region that is deleted in SJL mice. (*B*) Products from PCR reactions involving the Jβ2 3′ primer with Dβ2 (top panels) or Vβ12 (bottom panels) 5′ primers were gel-fractionated, Southern blotted, and hybridized with the Jβ2 probe. Sources of DNA are indicated above each lane; PCR products corresponding to the germ-line Dβ–Jβ cluster (GL) and specific rearrangements are indicated on the left. Serial 10-fold dilutions of wild-type thymocytes have been analyzed (top left); for each dilution the amount of template DNA was kept constant by adding E14.1 cells in the indicated proportions (100 corresponding to 2×10^3 cells). (*C*) Same as in *B* except that: (*i*) Sources of DNA were from offspring of either 129/Ola × SJL ($129^{wt}/SJL^{wt}$) or $\Delta E\beta^{-/-}$ × SJL ($129^{-}/SJL^{wt}$) intercrosses; (*ii*) 5′ primer was either Vβ12 (left) or Vβ14 (right). Stars indicate PCR products of unknown origin that were amplified from any tissue when using the Dβ2/Jβ2 or Vβ12/Jβ2 primer combinations.

shown), implying the lack of any TCR β gene rearrangement (and genuine $\alpha\beta^+$ cells) in the $\Delta E\beta^{-/-}$ mice.

Several types of TCR β transcripts have been described in immature T cells, including germ-line Vβ transcripts along with transcripts from partially (DJβ) or completely (VβDJβ) rearranged TCR β genes (19). To measure the relative levels of such transcripts in thymocytes from $\Delta E\beta^{-/-}$ mice, we used RT-PCR assays as previously described (14, 15). Examples of assays for transcripts containing Vβ5, Vβ11, and DJβ2 gene segments are shown in Fig. 5. Rearranged VβDJβ or DJβ transcripts were not detected in $\Delta E\beta^{-/-}$ thymocytes, whereas germ-line Vβ transcripts were readily found at a low level in these cells (Fig. 5, far right lane). When hybridizations on blots

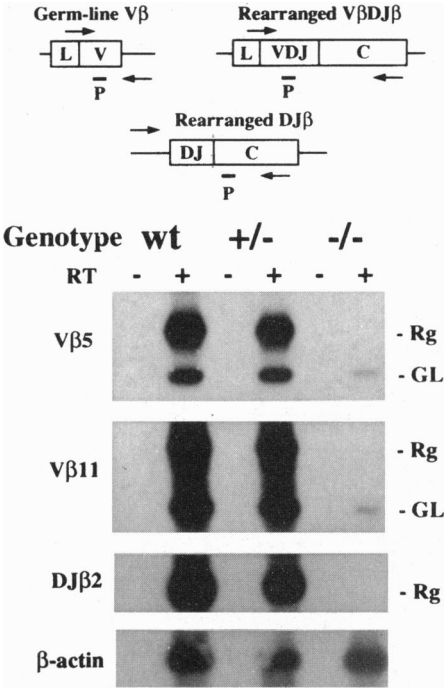

FIG. 5. TCR β gene expression in thymocytes from wild-type and mutant $\Delta E\beta^{+/-}$ or $\Delta E\beta^{-/-}$ mice. Germ-line (GL) Vβ and rearranged (Rg) VβDJβ or DJβ transcripts were analyzed using total RNA from 2×10^3 thymocytes. (*Top*) Diagram depicting the structure of the predicted PCR products along with the relative locations of the PCR primers (arrowheads) and hybridization probes (P). (*Bottom*) Products from PCR reactions were gel-fractionated, blotted, and hybridized with appropriate probes; Vβ analysis was performed by mixing 5′Vβ, 3′Vβ, and 3′Cβ2 primers in the same PCR reaction, then using Vβ-specific probe for hybridization. Samples were analyzed in which the reverse transcriptase (RT) was present (+) or not (−) in the reaction; each sample comprised a roughly equivalent amount of input RNA, as indicated by control β-actin PCRs.

similar to those shown in Fig. 5 were quantitated on a phosphoimager, Bio-Imaging analyzer BAS1000 (Fuji) the amounts of germ-line Vβ5 and Vβ11 transcripts found in $\Delta E\beta^{-/-}$ thymuses were calculated (after loading variations have been corrected for based on β-actin controls) to vary between 2% and 10% of those in thymuses from wild-type and heterozygous $\Delta E\beta^{+/-}$ littermates. These data would indicate that the $\Delta E\beta^{-/-}$ mutation does not completely abolish transcription at the TCR β gene loci. However, due to the altered T-cell differentiation in $\Delta E\beta^{-/-}$ thymuses, definitive conclusion about the extent of inhibition of TCR β transcription in the absence of Eβ awaits analysis of cells of matched surface phenotype; furthermore, transcription that goes through other TCR β gene segments, such as germ-line Jβ transcription, also needs to be analyzed (unpublished work).

In summary, using sensitive DNA-PCR and RT-PCR assays, we found no evidence of TCR β rearranged products in lymphoid cells from $\Delta E\beta^{-/-}$ mice; in T cells from heterozygous $\Delta E\beta^{+/}$ animals, β rearrangement appeared restricted to the nontargeted allele. Based on these findings and the lack of $\alpha\beta^+$ cells in $\Delta E\beta^{-/-}$ mice, we conclude that the Eβ enhancer is absolutely required for V(D)J cis-recombination to occur at the TCR β locus and completion of αβ T-lineage differentiation.

DISCUSSION

In this study, mice carrying a deletion of the Eβ enhancer were generated by gene targeting in ES cells. Mouse strains ($\Delta E\beta$-$neo^{-/-}$ and $\Delta E\beta^{-/-}$) homozygous for the Eβ mutation showed

a block of $\alpha\beta$ T-cell development resulting in the absence of $\alpha\beta^+$ thymocytes and lymph node T cells. A set of experimental results argued that altered T-cell development resulted from a lack of TCR β gene rearrangement in the absence of cis-acting Eβ sequences. These results illustrate the critical role that Eβ plays on the control of V(D)J recombination *in vivo* and provide further insights on the process of $\alpha\beta$ T-lineage differentiation.

In previous experiments (20, 21), deletion of the immunoglobulin heavy (IgH) intron enhancer was found to result in an incomplete block of cis-rearrangements in B-lineage cells, suggesting the existence of an additional cis-regulatory element(s) at the IgH locus. Likewise, although the replacement of the Igκ intron enhancer by a neo^r gene was reported to abolish cis-rearrangements (22), its deletion in the absence of any selectable marker gene (the most suitable condition for analyzing the effects of cis-regulatory elements; ref. 23) resulted in a less severe inhibition (F. W. Alt, personal communication). Without dismissing the possibility of additional β regulatory elements (24), our current results demonstrate that the functions on rearrangement of Eβ and these elements are not redundant. It is clear that Eβ is required for mediating accessibility of the Dβ and Jβ gene segments to the V(D)J recombinase. However, as D$\beta \rightarrow$ Jβ rearrangement precedes, and may be a prerequisite for V$\beta \rightarrow$ DJβ rearrangement, the role of Eβ in mediating accessibility of the Vβ segments remains uncertain. Analyses are in progress to address this issue as well as to determine whether a threshold of Eβ-dependent transcription needs to be reached for activation of cis-recombination or, alternatively, whether Eβ operates on recombination independently of its transcriptional enhancing function. In any case, the presence of germ-line Vβ transcription in $\Delta E\beta^{-/-}$ thymocytes is appealing in view to earlier findings that, in transgenic substrates, Eβ-induced transcription through β gene segments is not sufficient to induce their recombination (7, 25).

Thymic cell features in $\Delta E\beta^{-/-}$ mice, including interindividual variation of thymocyte numbers with few DP and SP TCR $\alpha\beta^-$ cells, suggest that cell development proceeds to some extent beyond the DN CD25$^+$ stage despite the TCR β rearrangement defect. Significantly, identical features are described in TCR β deficient (TCR$\beta^{-/-}$) mice (17). This common phenotype distinguishes the TCR$\beta^{-/-}$ and $\Delta E\beta^{-/-}$ knockout mice from RAG$^{-/-}$ or CD3ε, $\gamma,\delta^{-/-}$ mice (which lack DP and SP thymocytes; refs. 26–28) as well as from pT$\alpha^{-/-}$ mice (which have a few of both DP and SP TCR $\alpha\beta^+$ thymic cells; ref. 29). Given a model that postulates selection of CD25$^+$ cells based on the expression of a pre-TCR, incomplete versus complete block of development at the DN CD25$^+$ stage may, in the first place, depend on whether $\gamma\delta^+$ thymocytes are produced (in TCR$\beta^{-/-}$, pT$\alpha^{-/-}$, and E$\beta^{-/-}$ mice) or not (in RAG$^{-/-}$ and CD3$\varepsilon\gamma\delta^{-/-}$ mice) (refs. 17 and 26–29; this study). When present $\gamma\delta^+$ thymocytes may trigger, possibly through interactions with the thymic stroma, a pre-TCR independent maturation pathway thus promoting the transition from DN to DP cells with a low efficiency (5, 30). Assuming that functionally rearranged TCR α chains are produced in DP cells that might have developed in this way (ref. 17; unpublished results), their behavior would then depend on whether TCR β chains are available or not. In $\Delta E\beta^{-/-}$ and TCR$\beta^{-/-}$ mice, such cells lacking TCR β chains do not differentiate further and are eliminated. Our observations in $\Delta E\beta^{-/-}$ thymuses of a differentiated, yet disorganized thymic medulla and of low CD8/CD4 SP and DP expressing cells (presumably, cells on the way to the DP stage and pre-apoptotic DP cells, respectively) are other experimental arguments supporting the above model of an alternate, pre-TCR independent maturation pathway.

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